Eur J Nutr (2013) 52:1169–1179
DOI 10.1007/s00394-012-0427-0

ORIGINAL CONTRIBUTION

Milk phospholipid and plant sterol-dependent modulation of plasma lipids in healthy volunteers

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Received: 22 March 2012 / Accepted: 16 July 2012 / Published online: 27 July 2012

Abstract
Purpose Hypolipidemic and/or hypocholesterolemic effects are presumed for dietary milk phospholipid (PL) as well as plant sterol (PSt) supplementation. The aim was to induce changes in plasma lipid profile by giving different doses of milk PL and a combination of milk PL with PSt to healthy volunteers.
Methods In an open-label intervention study, 14 women received dairy products enriched with moderate (3 g PL/day) or high (6 g PL/day) dose of milk PL or a high dose of milk PL combined with PSt (6 g PL/day + 2 g PSt/day) during 3 periods each lasting 10 days.
Results Total cholesterol concentration and HDL cholesterol concentration were reduced following supplementation with 3 g PL/day. No significant change in LDL cholesterol concentration was found compared with baseline. High PL dose resulted in an increase of LDL cholesterol and unchanged HDL cholesterol compared with moderate PL dose. The LDL/HDL ratio and triglyceride concentration remained constant within the study. Except for increased phosphatidyl ethanolamine concentrations, plasma PL concentrations were not altered during exclusive PL supplementations. A combined high-dose PL and PSt supplementation led to decreased plasma LDL cholesterol concentration, decreased PL excretion, increased plasma sphingomyelin/phosphatidyl choline ratio, and significant changes in plasma fatty acid distribution compared with exclusive high-dose PL supplementation.
Conclusion Milk PL supplementations influence plasma cholesterol concentrations, but without changes of LDL/HDL ratio. A combined high-dose milk PL and PSt supplementation decreases plasma LDL cholesterol concentration, but it probably enforces absorption of fatty acids or fatty acid-containing hydrolysis products that originated during lipid digestion.

Keywords Sphingomyelin · Phosphatidyl choline · Cholesterol · Bile acids · Fatty acids · Dairy products

Abbreviations

CD36	Cluster of differentiation 36
DM	Dry matter
FA	Fatty acid
FABP	Fatty acid binding protein
FAME	Fatty acid methyl ester
MUFA	Mono-unsaturated fatty acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PI	Phosphatidyl inositol
PL	Phospholipid
PSt	Plant sterol
PUFA	Poly-unsaturated fatty acid
SFA	Saturated fatty acid
SM	Sphingomyelin
SMFA	Milk sphingomyelin-related fatty acid
SR-BI	Scavenger receptor class B type I
TG	Triglyceride

Electronic supplementary material The online version of this article (doi:10.1007/s00394-012-0427-0) contains supplementary material, which is available to authorized users.

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Introduction

Bovine milk lipids consist of about 95 % triglycerides (TGs) as a main component in addition to approximately 1 % phospholipids (PLs). The milk PLs are composed of a mixture of glycero PLs such as phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE), as well as sphingo PLs, such as sphingomyelin (SM). PC, PE, and SM are present in almost equal ratio with 30 % of total PLs [1]. The fatty acid (FA) distribution in bovine milk PL is dominated by saturated FAs (SFAs; PC $\approx$ 50; PE $\approx$ 30; SM $\approx$ 90; [wt%]). Moreover, PE is composed of a large fraction of mono-unsaturated FAs (MUFAs; 45 wt%) and, in addition, a characteristic of bovine SM is a high proportion of very long FAs (Σ C22:0, C23:0, C24:0, C24:1 = 53 wt%) [1, 2]. The content of poly-unsaturated FAs (PUFAs) in SM, PC, and PE amounts to about 1, 15, and 25 wt% respectively [1].

Data describing the mechanism for intestinal PL absorption are rare. PC is the most abundant luminal PL comprising of biliary (10–12 g/day) and dietary PC (1–2 g/day) [3, 4]. With reference to glycero PLs, it is known that the pancreatic enzyme phospholipase A_2 catalyzes the cleavage of PL into lyso-PL and free FA at the β-carbon atom [5]. Studies in pancreatic phospholipase A_2-deficient mice demonstrate that in addition to the pancreatic phospholipase A_2 other phospholipases are also necessary for PL hydrolysis [6]. SM is hydrolyzed into ceramides or sphingoid bases by SMases and ceramidases during digestion. Although the initial process of molecular uptake of sphingolipids into the enterocytes is not clear, once inside the enterocytes, the sphingoid bases are mainly metabolized to FAs [7, 8]. Together with bile, the lipid degradation products (lyso-PLs, FAs, monoglycerides, diglycerides, etc.) form mixed micelles in the lumen, which are essentially required for the absorption of apolar lipids [9].

To date, information relating to the influence of dietary supplementation of a glycero and sphingo PL mixture, in particular milk PLs, on plasma lipid concentrations is scant. However, animal studies in C57BL/6 mice receiving a high-fat diet combined with milk PLs indicated reduced hepatic and plasma concentrations of PLs, TGs, and cholesterol compared with feeding a high-fat diet alone. No differences in hepatic and plasma lipid concentrations occurred on using a normal non-purified diet as basal diet [10, 11].

Regarding the milk PL components PE, PC, and SM, a few in vivo studies have been carried out that investigated a PL influence either on intestinal cholesterol absorption or reduction of plasma cholesterol. Feeding a PE-rich diet to minks led to reduced plasma PL and cholesterol concentrations and increased fecal PL and cholesterol excretions compared with feeding a diet based on soybean oil [12]. Egg PC, rich in SFA, was found to decrease the lymphatic cholesterol absorption in rats compared with soy PC [13]. Further, sphingolipid application to APO*3Leiden mice resulted in a decreased serum cholesterol concentration and indicated an inhibited cholesterol absorption [14]. Moreover, one study in rats revealed that a combined supplementation of SM and cholesterol decreased the absorption of both compounds. The same reduction in SM absorption was found on replacing cholesterol with the plant sterol (PSt) sitostanol [15].

A cholesterol-lowering effect of a PSt supplementation was verified in several studies, in which a supplementation with 1.5–2 g/day led to a reduction in LDL cholesterol between 10 and 15 % [16]. A combination of in vivo studies demonstrated that this decrease in plasma LDL cholesterol concentration is dependent on the basal fractional cholesterol synthesis [17]. The human studies of Rideout et al. [17] showed an increase in plasma LDL cholesterol concentration of 3.7 % following PSt supplementation in 47 non-responders with a high cholesterol fractional synthesis rate. However, studies in mice, also non-responding to dietary PSt, did not verify an increase in non-HDL cholesterol after PSt feeding [17]. In the case of response to dietary PSt, reduced plasma cholesterol level is probably due to a competitive intestinal absorption of the structurally similar compounds [16]. A combined supplementation of milk PL and PSt may alter the physical conditions of the luminal micellar system and result in intestinal interactions of either the two compounds between themselves and/or with cholesterol.

To investigate the hypolipidemic and/or hypocholesterolemic effects associated with dietary milk PLs or milk PL components found in animal studies and to examine an impact of additional PSt supplementation on plasma lipid status, healthy volunteers were given different doses of milk PLs: moderate dose (3 g PL/day), high dose (6 g PL/day), and a high dose of milk PL combined with PSts (6 g PL + 2 g PSt/day) with an aim to induce changes in the plasma lipid profile (cholesterol, PLs, TGs, FAs).

Materials and methods

Participants

The study was approved by the ethics committee of the Friedrich Schiller University Jena and was registered under the Clinical Trials Registry (NCT 01327430). All participants were informed about the study conditions in both verbal and written form. In addition, written consent was given by participating subjects. Fifteen healthy female volunteers were recruited at the start of the study, though one

subject was excluded because of a relevant hypertriglyceridemia with a plasma TG concentration of 6.71 mmol/L. Subjects were 36 ± 5 years old and had a BMI of 24.8 ± 3.9 kg/m^2. Exclusion criteria were metabolic diseases as well as a suggested or verified arteriosclerosis.

Study design

The study consisted of 3 supplementation periods, each lasting for 10 days. During the first period, volunteers received a daily dose of 3-g milk PLs ("3 g PL"). In the second period, the milk PL intervention was doubled to 6 g per day ("6 g PL") and for the third period, the high PL dose was combined with a PSt supplement of 2 g per day ("6 g PL + 2 g PSt"). At study begin ("baseline") and at the end of each period, all volunteers completed a 3-day dietary record. In addition, subjects collected their complete feces during these 3 days. The samples were weighed daily and kept frozen (− 20 °C) until homogenization and lyophilization. After the 3 days of dietary record and feces collection, fasting blood samples were taken using lithium-heparin monovettes. Following centrifugation (2,500 ×*g*, 10 min), the plasma was separated, aliquoted, and stored frozen (−20 °C) until analysis.

Test products

The PL concentrate (Lipamin M20, charge: 0000258288, Uelzena eG, Uelzen, Germany) was prepared from butter serum and contained 52 % total protein, 5 % lactose, and 38 % total fat as determined by extraction with a 2-propanol/n-hexane (2:3, v:v) mixture. Total PL content was found to be 18.4 % (PC: 5.2 %, PE: 4.8 %, SM: 4.3 %, phosphatidyl serine: 2.1 %, phosphatidyl inositol (PI): 1.5 %, other: 0.5 %). To supply the PL concentrate in the form of a consumable food matrix, it was added and mixed into two different dairy products: bovine milk with a fat content of 1.5 % and a low-fat curd (0.1 % fat) flavored with fresh herbs (Table 1). The daily intake of 2 g PSt was realized by an intake of a commercial PSt drink.

Plasma cholesterol

Total cholesterol, LDL cholesterol, HDL cholesterol, and TG concentrations in plasma were photometrically analyzed using the ARCHITECT® system (Abbott Laboratories, IL, USA) according to the manufacturer's recommendations.

Plasma phospholipids

Total plasma fat was quantified in duplicate with aliquot volumes of 1 mL plasma using gravimetry based on the lipid extraction method described by Bligh and Dyer [18]. The lipid extract was applied for determining the individual PL concentrations using a densitometric HPTLC method that combined the methods of Xu et al. [19], Colarow [20], and Lendrath et al. [21]. In short, an aliquot of the lipid extract was dissolved in chloroform (ca. 0.2 %, wt:v) and 15 µL was sprayed on a HPTLC plate (silica gel 60, F 254, Merck KGaA, Darmstadt, Germany) employing an autosampler Linomat IV (Camag, Muttenz, Switzerland). Analyte separation was realized using two different mobile phases. The first separation was carried out with n-hexane:diethyl ether = 60:40 (v:v); the second involved a five component mixture consisting of chloroform:methanol:acetone:aqua dest.:glacial acetic acid = 44:32:17.5:5:5 (v:v:v:v:v). Thereafter, the PLs were derivatized using diluted phosphoric acid containing copper(II) sulfate, and developed for 20 min at 180 °C. Finally, they were detected at 400 nm on a densitometer (TLC Scanner 3, Camag, Muttenz, Switzerland). Standard substances for

Table 1 Intake of provided dairy products and supplemented PLs following daily supplementation with moderate dose (3 g PL), high dose (6 g PL), and high dose of milk phospholipids combined with plant sterols (6 g PL + 2 g PSt)

	Baseline	3 g PL	6 g PL	6 g PL + 2 g PSt
Milk, 1.5 % fat (mL/day)	–	300	300	300
PL concentrate intake (g/day)	–	16.2	16.2	16.2
PL intake from PL concentrate (g/day)	–	2.98	2.98	2.98
PC/PE/SM intake (g/day)	–	0.84/0.78/0.70	0.84/0.78/0.70	0.84/0.78/0.70
Curd, 0.1 % fat (g/day)	–	100	100	100
PL concentrate intake (g/day)	–	–	16.2	16.2
PL intake from PL concentrate (g/day)	–	–	2.98	2.98
PC/PE/SM intake (g/day)	–	–	0.84/0.78/0.70	0.84/0.78/0.70
Total PL intake (g/day)		2.98	5.96	5.96
Total PC/PE/SM intake (g/day)		0.84/0.78/0.70	1.68/1.56/1.40	1.68/1.56/1.40

PC phosphatidyl choline, *PE* phosphatidyl ethanolamine, *PL* phospholipid, *PSt* plant sterol, *SM* sphingomyelin

qualitative and quantitative analysis of lyso-PC, PC, SM, PE, and PI were purchased from Sigma-Aldrich, Taufkirchen, Germany. For data validation, total PLs in plasma were determined using a second approach, which was modified according to the method of Rouser et al. [22]. Briefly, the lipid extract from 1 mL plasma was oxidized with perchloric acid. Addition of ammonium hepta molybdate and ascorbic acid formed the dye molybdenum blue, which is photometrically detectable at 820 nm. To calculate the PL content, a factor of 25.0 was used, which is in accordance with Goodman and Shiratori [23] and which represents a close average of other earlier described PL data (25.4, 24.4) [24, 25]. Comparison of the total PL concentrations in 42 plasma samples revealed no differences between both methods (HPTLC method: 2.29 ± 0.38 mg/mL *versus* molybdenum blue method: 2.26 ± 0.37 mg/mL).

Plasma fatty acids

The total FA distribution in plasma was analyzed by derivatization of the plasma FAs to methyl esters (FAMEs) with methanolic hydrochloric acid (1 h, 80 °C). FAMEs were purified by TLC and finally, separated and detected by GC-FID (GC17A, Shimadzu, Kyoto, Japan; column: DB225 ms, 60 m length, 0.25 mm diameter, 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). Reference standards were used as FAME mixture to identify FA peaks (Nu-Chek Prep Inc., Elysian, MN, USA; Larodan, Malmö, Sweden). The FAs were divided in SFAs (Σ C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C23:0, C24:0), MUFAs (Σ C16:1Δ9c, C18:1Δ9t, C18:1Δ9c, C18:1Δ11c, C20:1Δ11c, C22:1Δ13c, C24:1Δ15c), PUFAs (Σ *all-cis*-C18:2Δ9,12, *all-cis*-C18:3Δ6,9,12, *all-cis*-C18:3Δ9,12,15, C18:2Δ9c,11t, *all-cis*-C20:2Δ11,14, *all-cis*-C20:3Δ8,11,14, *all-cis*-C20:5Δ5,8,11,14,17, *all-cis*-C22:4Δ7,10,11,13,16, *all-cis*-C22:5 Δ4,7,10,13,16, *all-cis*-C22:5Δ7,10,13,16,19, *all-cis*-C22:6Δ4,7,10,13,16,19), and according to Valeur et al. [26] in milk SM-related FAs (SMFA; Σ C22:0, C23:0, C24:0, C24:1Δ15c) as marker for compliance with study conditions.

Feces parameters

Fecal fat was determined gravimetrically after acidic feces decomposition and lipid extraction employing the Soxhlet apparatus with petrol ether. Sterols and bile acids from feces were prepared and quantified as described earlier [27]. Fecal cholesterol concentration represents the sum of cholesterol, coprostanol, coprostanon, cholestanol, and cholestanon. Fecal bile acid concentration represents the sum of cholic acid, chenodeoxycholic acid, deoxycholic acid, iso-deoxycholic acid, 12-keto-deoxycholic acid, lithocholic acid, and iso-lithocholic acid. Fecal total PL concentration was analyzed in the lipid extract [18] using the method forming molybdenum blue. Hence, phosphorous-free luminal degradation products of PLs such as ceramides and sphingoid bases are not considered in the fecal total PL concentration. To calculate the PL content, the factor of 25.0 was used.

Data handling and statistical methods

FA, sterol, and bile acid chromatogram analyses were conducted employing GCsolution® and GCMSsolution® softwares (Shimadzu, Kyoto, Japan). PL chromatograms were evaluated using the TLC software CATS® (Camag, Muttenz, Switzerland). Evaluation of the dietary records was carried out using PRODI® (5.0 expert, Nutri-Science, Freiburg, Germany). Statistical analyses of the experimental results were generated utilizing IBM SPSS Statistics® (version 19.0, IBM Corporation, Armonk, NY, USA) with the general linear model for a repeated measures design. Correlations were calculated using the Pearson coefficient. The statistical test criteria for a probability level (P) less than 0.05 were necessary for a sample value difference to be considered as significant. The results were stated as mean ± standard deviation (SD).

Results

Base parameters

The energy intake calculated from the 3-day dietary records was in the region of 8 and 10 MJ/day in all study periods, in compliance with the 138–163 kJ/kg value recommended for women with a mean age of 36 years, a body mass of 72 ± 12 kg, and a low-to-middle degree of physical activity (Table 2) [28]. The additional energy intake during daily supplementation with 3 g PLs due to the PL concentrate amounts to approximately 400 kJ/day, whereas the value for the other two period was around 800 kJ/day. The content of total plasma fat remained unchanged during the whole study (baseline: 5.69 ± 0.79; 3 g PL: 5.73 ± 0.78; 6 g PL: 5.71 ± 1.08; 6 g PL + 2 g PSt: 5.88 ± 0.79; [mg/mL]).

Daily stool excretion tended to be higher or was significantly higher during the 6 g PL supplementation periods compared with baseline. The daily excretion of fat was unchanged throughout the study. However, a slight PL dose-dependent increase of fat excretion is distinguishable with a reduction on PSt supplementation. There was a significant increase of fecal pH in all intervention periods in comparison with baseline. No significant differences in

Table 2 Base parameter of intake and excretion at baseline and following daily supplementation with moderate dose (3 g PL), high dose (6 g PL), and high dose of milk phospholipids combined with plant sterols (6 g PL + 2 g PSt)

	Baseline	3 g PL	6 g PL	6 g PL + 2 g PSt
Intake				
Energy (MJ/day)[a]	8.21 ± 1.63	9.13 ± 2.09	8.46 ± 2.68	9.55 ± 2.14*
Total fat intake (g/day)[a]	66 ± 16	71 ± 20	69 ± 22	76 ± 15
Fat intake from PL product (g/day)	–	6.2	12.8	12.8
Excretion				
Fresh stool (g/day)	114 ± 53	115 ± 41	139 ± 49*	134 ± 37
Fecal fat (g/day)	4.3 ± 1.8	5.1 ± 2.7	7.2 ± 3.5	6.8 ± 2.5
Fecal pH	6.44 ± 0.30	6.79 ± 0.31*	6.73 ± 0.29*	6.75 ± 0.27*

Statistical analysis was performed with the general linear model for a repeated measures design. Data are expressed as mean ± SD. $n = 14$

PL phospholipid, *PSt* plant sterol

*$P < 0.05$ versus baseline

[a] Result obtained from 3-day dietary records, which include intake from dairy products (300 mL milk and 100 g curd), but not from the PL concentrate

fecal dry matter (DM), proportion of fat, and the protein content were verified (data are not shown).

Phospholipid parameters

Total PL and PC concentration in plasma were unchanged between all periods (Table 3). However, the percent PC proportion of total PLs was significantly reduced after supplementation with 6 g PL + 2 g PSt as compared with all other periods (Fig. 1). In contrast, in the same period, the percent SM and lyso-PC proportions were significantly increased in comparison with all other periods. Additionally, the absolute concentrations of SM and lyso-PC in plasma were increased when assessed against baseline values and also compared with the daily dose of 3 g PL. These changes resulted in significantly higher SM/PC as well as SM/(SM + PC) ratios in the supplementation period with 6 g PL + 2 g PSt in comparison with all other periods.

There was no correlation between lyso-PC and PC plasma concentrations. Interestingly, there was a positive correlation between SM and PC plasma concentrations at baseline ($n = 14$, $r = 0.63$, $P = 0.016$) and after supplementation with 6 g PL + 2 g PSt ($n = 14$, $r = 0.67$, $P = 0.009$). However, SM and PC plasma concentration showed no correlation after supplementation with 3 g PL and 6 g PL. The concentration of PE in plasma was increased in all supplementation periods in comparison with baseline and showed significance after supplementation with 3 g PL and 6 g PL + 2 g PSt.

Fecal concentration of total PLs increased in a dose-dependent manner with significantly higher fecal PL contents after PL supplementations in comparison with baseline. The combination of 6 g PL and 2 g PSt resulted in a significant lower fecal PL concentration than after supplementation with 6 g PL alone.

Cholesterol parameters

Total cholesterol concentration in plasma was significantly lower after a moderate dose of 3 g PL in comparison with baseline due mainly to a reduction of HDL cholesterol concentration (Table 4). Following supplementation with 6 g PL, the total cholesterol concentration rose significantly compared with supplementation with 3 g PL as a result of a significant increase of LDL cholesterol concentration. Combined high-dose PL and PSt supplementation resulted in a significantly lower LDL cholesterol concentration compared with supplementation of only highly dosed PL. No changes in the ratio of LDL to HDL cholesterol were verified during the study. Additionally, no differences between the plasma TG concentrations due to the different supplementations were observed. However, a slight but constant increase of plasma TG concentration during the course of the study was noticed.

Fecal cholesterol concentration was significantly lower after supplementation with 3 g PL compared with the two high PL supplementation periods. In contrast, fecal bile acid concentration was highest after the moderate PL dose, showing a significant difference in comparison with baseline and with supplementation with 6 g PL + 2 g PSt.

Fatty acid parameters

The proportion of SFAs in plasma rose significantly during the study (Fig. 2). Interestingly, the proportion of MUFAs was similarly increased for both PL supplementation periods in comparison with baseline. Due to the combination of

Table 3 Phospholipid parameter of plasma and feces at baseline and following daily supplementation with moderate dose (3 g PL), high dose (6 g PL), and high dose of milk phospholipids combined with plant sterols (6 g PL + 2 g PSt)

	Baseline	3 g PL	6 g PL	6 g PL + 2 g PSt
Plasma				
Total PL (mg/mL)	2.23 ± 0.34	2.27 ± 0.44	2.35 ± 0.36	2.29 ± 0.41
Lyso-PC (mg/mL)	0.07 ± 0.04	0.08 ± 0.03	0.08 ± 0.05	0.10 ± 0.03*,#
PC (mg/mL)	1.58 ± 0.24	1.59 ± 0.36	1.65 ± 0.30	1.54 ± 0.32
SM (mg/mL)	0.46 ± 0.08	0.45 ± 0.10	0.48 ± 0.07	0.51 ± 0.09*,#
PE (mg/mL)	0.09 ± 0.04	0.12 ± 0.05*	0.11 ± 0.05	0.12 ± 0.04*
PI (mg/mL)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
PE/PC	0.057 ± 0.019	0.070 ± 0.021*	0.066 ± 0.024	0.075 ± 0.014*
SM/PC	0.29 ± 0.04	0.30 ± 0.08	0.30 ± 0.05	0.34 ± 0.05*,#,+
SM/(SM + PC)	0.23 ± 0.02	0.23 ± 0.05	0.23 ± 0.03	0.25 ± 0.03*,#,+
Feces				
Total PL (mg/g DM)[a]	9.03 ± 5.06	12.8 ± 5.9*	15.8 ± 8.9*	10.3 ± 4.0+

Statistical analysis was performed with the general linear model for a repeated measures design. Data are expressed as mean ± SD. $n = 14$

DM dry matter, *PC* phosphatidyl choline, *PE* phosphatidyl ethanolamine, *PI* phosphatidyl inositol, *PL* phospholipid, *PSt* plant sterol, *SM* sphingomyelin

* $P < 0.05$ versus baseline; #$P < 0.05$ versus 3 g PL; +$P < 0.05$ versus 6 g PL

[a] The phosphorous-free luminal degradation products of PLs like ceramides, sphingoid bases, monoglycerides, and diglycerides are not considered in the fecal total PL concentration due to the analytical conditions

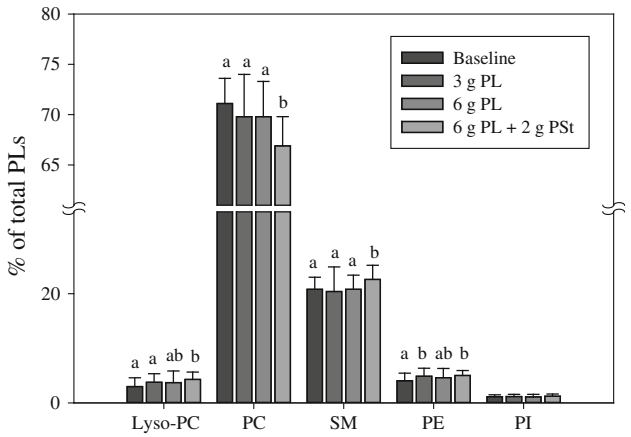

Fig. 1 Phospholipid pattern of plasma at baseline and following daily supplementation with moderate dose (3 g PL), high dose (6 g PL), and high dose of milk phospholipids combined with plant sterols (6 g PL + 2 g PSt). Statistical analysis was performed with the general linear model for a repeated measures design. Data are expressed as mean ± SD. $n = 14$. *Different letters indicate significant differences between study periods,* $P < 0.05$. *PC* phosphatidyl choline, *PE* phosphatidyl ethanolamine, *PI* phosphatidyl inositol, *PL* phospholipid, *PSt* plant sterol, *SM* sphingomyelin

PL and PSt, the MUFA fraction—mainly C18:1Δ9c—increased compared with both PL periods. The same conditions were found while examining the fraction of SMFAs. On the contrary, the proportion of PUFAs in plasma decreased during both PL supplementation periods compared with baseline. Moreover, the addition of PSt led to the significantly lowest PUFA concentration within the whole study, which is predominantly a result of a decrease of *all-cis*-C18:2Δ9,12. Although, fecal SFA and PUFA proportions were not significantly changed during the study, the fraction of MUFA was reduced in the period of supplementation with 6 g PL + 2 g PSt compared with all other periods. The proportion of fecal SMFAs rose significantly during the course of the study.

Correlations between plasma/feces and feces/feces parameters

Considering data from all study periods, total plasma cholesterol as well as LDL concentration correlated negatively with fecal bile acid excretion on the base of statistical significance ($P < 0.05$) or by a trend ($P < 0.1$). A significantly positive correlation was found between plasma TG concentration and cholesterol excretion ($P = 0.003$) as well as PL excretion ($P = 0.028$). No correlation was found between changes in plasma LDL levels and changes in fecal cholesterol or PL excretion. Alterations in fecal lipid excretion were rather associated with changes in plasma HDL concentration.

There was a significant positive association of fat excretion with cholesterol, bile acid, and PL excretion. Although the PL and cholesterol excretion correlated significantly positive ($P = 0.000$), the changes in PL and cholesterol excretion between the periods in order of the study (baseline → 3 g PL → 6 g PL) did not correlate ($P = 0.821$). (Correlation data are attached as supplementary material.)

Table 4 Cholesterol and bile acid parameter of plasma and feces at baseline and following daily supplementation with moderate dose (3 g PL), high dose (6 g PL), and high dose of milk phospholipids combined with plant sterols (6 g PL + 2 g PSt)

	Baseline	3 g PL	6 g PL	6 g PL + 2 g PSt
Plasma				
Total cholesterol (mmol/L)	5.12 ± 0.63	4.72 ± 0.71*	5.07 ± 0.79$^{\#}$	4.87 ± 0.67*
LDL cholesterol (mmol/L)	2.71 ± 0.77	2.54 ± 0.69	2.86 ± 0.84$^{\#}$	2.59 ± 0.75^{+}
HDL cholesterol (mmol/L)	1.76 ± 0.47	1.62 ± 0.40*	1.68 ± 0.42	1.62 ± 0.44*
LDL/HDL ratio	1.74 ± 1.02	1.72 ± 0.86	1.86 ± 0.93	1.81 ± 0.97
Triglycerides (mmol/L)	1.06 ± 0.21	1.08 ± 0.30	1.11 ± 0.36	1.14 ± 0.37
Feces				
Cholesterol (mg/g DM)[a]	21.7 ± 6.5	20.5 ± 5.2	24.4 ± 5.2$^{\#}$	23.1 ± 5.5$^{\#}$
Bile acids (mg/g DM)[b]	8.89 ± 2.77	10.1 ± 3.9*	9.29 ± 3.56	8.55 ± 3.13$^{\#}$

Statistical analysis was performed with the general linear model for a repeated measures design. Data are expressed as mean ± SD. $n = 14$

DM dry matter, *PL* phospholipid, *PSt* plant sterol

* $P < 0.05$ versus baseline. $^{\#}P < 0.05$ versus 3 g PL. $^{+}P < 0.05$ versus 6 g PL

[a] Fecal cholesterol concentration represents the sum of cholesterol, coprostanol, coprostanon, cholestanol, and cholestanon

[b] Fecal bile acid concentration represents the sum of cholic acid, chenodeoxycholic acid, deoxycholic acid, iso-deoxycholic acid, 12-keto-deoxycholic acid, lithocholic acid, and iso-lithocholic acid

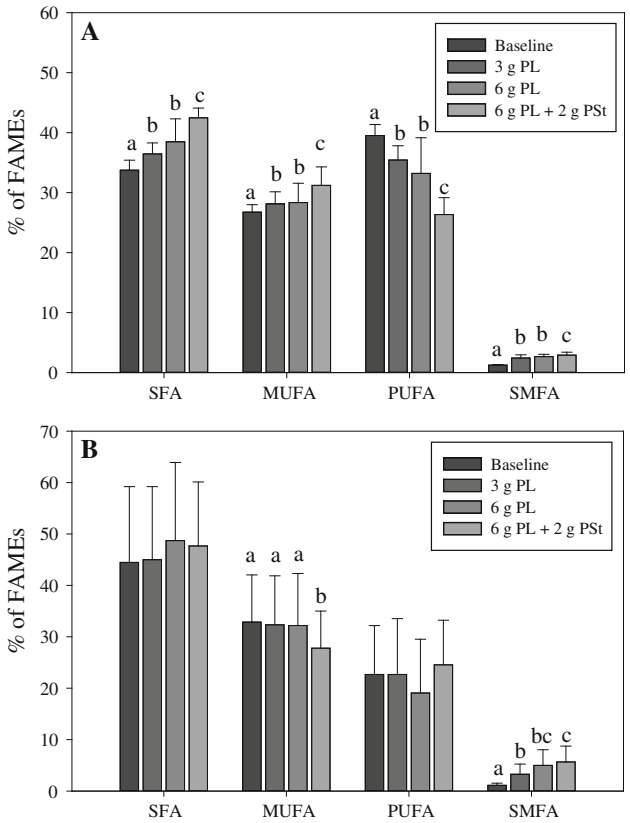

Fig. 2 Fatty acid pattern of plasma (**a**) and feces (**b**) at baseline and following daily supplementation with moderate dose (3 g PL), high dose (6 g PL), and high dose of milk phospholipids combined with plant sterols (6 g PL + 2 g PSt). Statistical analysis was performed with the general linear model for a repeated measures design. Data are expressed as mean ± SD. $n = 14$. *Different letters indicate significant differences between study periods, $P < 0.05$. PL* phospholipid, *PSt* plant sterol, *SMFA* milk sphingomyelin-related fatty acid

Discussion

On the basis of animal studies showing hypolipidemic and/or hypocholesterolemic effects of milk PLs, milk PL components, and PSts, a human study with healthy volunteers receiving different doses of milk PL as well as a combination of milk PL and PSt was carried out. Although total cholesterol concentration and HDL cholesterol concentration were reduced following daily supplementation with 3 g PL, no significant changes in LDL cholesterol concentration were found compared with baseline. Doubling the daily dose to 6 g PL resulted in a significant increase of LDL cholesterol compared with supplementation with 3 g PL. The ratio of LDL to HDL cholesterol was unchanged during the course of the study. The combination of 6 g PL + 2 g PSt led to a significant reduction of LDL cholesterol in plasma compared with supplementation with 6 g PL alone.

Studies in C57BL/6 mice fed milk PLs in combination with a high-fat diet indicated reduced plasma or liver lipid levels compared with animals receiving a high-fat diet only [10, 11]. In our study with healthy volunteers, no changes in LDL cholesterol concentration in plasma were found following supplementation with 3 g milk PLs per day and a daily intake of about 9.1 MJ/d compared with baseline, which is in accordance to the results of both an animal study feeding milk PLs on the basis of a normal diet [11] and a human study with 48 subjects, within which no changes in the plasma lipid concentration following a daily consumption of 3 g butter milk PLs were seen over a 4-week period [29].

Following an intake of dairy products, contradictory results reporting increased as well as decreased LDL cholesterol concentrations have been described by some

authors [30–32]. The increase in LDL cholesterol linked with consumption of dairy products was associated with a high intake of SFA found in milk fat [30]. Distribution of FAs in the PL concentrate applied in this study comprised 58 % SFA, 32 % MUFA, and 8 % PUFA. Increased fractions of SFA and MUFA as well as a decrease in the proportion of PUFA were demonstrated in plasma after consumption of moderate and high PL dose in comparison with baseline. Additionally, plasma LDL cholesterol concentration rose following supplementation with a high dose of PL possibly due to an increased intake of SFA. However, no significant alterations in plasma TG concentrations were found following PL supplementation compared with baseline.

The hypocholesterolemic effects of dietary PLs are described as a possible result of intestinal interactions between PLs and cholesterol. PC rich in SFA that originated from eggs was found to lower the intestinal absorption of cholesterol in rats [13]. Moreover, SM and cholesterol have a high affinity [33] and probably form cholesterol-SM complexes in the intestine. This interaction may influence the intestinal solubility of both components as well as their degree of absorption as seen in studies with rats [15]. Accordingly, inhibited cholesterol absorption in the intestine following milk PL absorption should result in increased cholesterol excretion. Although we found a highly significant positive correlation between daily PL and cholesterol excretions, no correlation could be verified with respect to changes in PL and cholesterol excretion in order of baseline → 3 g PL → 6 g PL. In addition, plasma LDL cholesterol concentration was only associated with bile acid excretion. Furthermore, changes in plasma HDL concentration were linked more to alteration in fecal lipid excretion, which is of interest as Wat et al. [11] demonstrated an effect of dietary milk PLs on HDL cholesterol in C57BL/6 mice. Altogether, the current data indicate a more significant influence of hepatic regulation of plasma cholesterol than an effect of inhibited cholesterol absorption in the intestine during milk PL supplementation. Hepatic regulation of plasma cholesterol due to PC synthesis from PE via PE methylation was also concluded after feeding a PE-rich diet to minks [12].

Although there were no changes in plasma PC and SM concentrations, PE concentration and PE/PC ratio were significantly increased following 3 g PL supplementation as well as 6 g PL + PSt supplementation compared with baseline. Various explanations are possible for the increased plasma PE concentrations and PE/PC ratios following milk PL supplementation in this study, such as (1) the direct influence of dietary PE in accordance with a study in which minks were nourished with fats high in PE [12], (2) the indirect influence of dietary SM due to increased sphingosine catabolism into ethanolamine phosphate enhancing PE biosynthesis as demonstrated in a study in rats fed with PE-free sphingolipids [34, 35], (3) the complex interactions between PE and PC in PL metabolism dependent, for example, on diglycerides, choline, or methionine [36], or (4) an increased PE biosynthesis due to increased oleic acid (C18:1Δ9c) proportion in plasma as demonstrated in an in vitro study with hepatocytes [37]. The proportion of oleic acid in plasma was significantly increased in the current study following supplementation with 3 g PL and 6 g PL + PSt in comparison with baseline (baseline: 21.7 ± 0.9; 3 g PL: 23.0 ± 1.9; 6 g PL: 22.9 ± 2.4; 6 g PL + 2 g PSt: 25.2 ± 2.5; [% oleic acid of total FAME]).

The majority of changes in the plasma lipid profile occurred following supplementation with combined high PL dose and PSt. A significant reduction in plasma LDL cholesterol concentration in the presence of unchanged HDL cholesterol concentration was observed after consumption of 6 g PL + 2 g PSt compared with only 6 g PL. PSts compete with cholesterol for incorporation into luminal micelles and for transfer into the enterocytes via the transporter Niemann-Pick C1 like 1. Additionally, unesterified PSts are re-transported from the enterocyte into the lumen via the ABC transporter G5 and G8 [38]. LDL cholesterol reducing effects in plasma following PSt supplementation have often been described. Although on the one hand, a resulting decreased risk for development of cardiovascular diseases has been suggested [39], on the other hand, a great deal of discussion has been raised regarding a proatherogenic potential of elevated plasma PSt concentration. The background of this discussion relates to different observations and findings such as (1) atherosclerotic diseases in patients suffering from phytosterolemia, (2) data from case report, clinical, genome-wide association, and epidemiological studies in humans, (3) results from animal studies indicating an accumulation of PSt following dietary supplementation or a facilitation of cholesterol transfer into atherosclerotic lesions by phytosterols [40–47]. Furthermore, the SM/(SM + PC) ratio in plasma has been suggested as being a predictive value for cardiovascular disease [48]. This ratio was significantly increased following combined PL and PSt supplementation in comparison with all other periods and the concentration of SM in plasma was highest following combined supplementation with 6 g PL + 2 g PSt. Increased plasma SM concentration in healthy subjects occurs with a large lipid load and an increase of TG-rich lipoproteins as a consequence of SM resistance to lipases and lecithin-cholesterol acyltransferase [49, 50]. In this study, no significant change in plasma TG concentration was found.

Besides the increase on SFA/MUFA and the decrease of PUFA proportions in plasma following PL supplementation, these changes further are fortified by combined

supplementation of 6 g PL and 2 g PSt. However, nearly 60 % of FAs in plasma cholesterol esters are PUFAs [51, 52]. Therefore, reduction of the PUFA level in plasma due to high SFA/low PUFA load via milk fat seems to be reinforced by a decrease of cholesterol ester concentration following combined supplementation with 6 g PL + 2 g PSt compared with 6 g PL alone. Nevertheless, it was demonstrated that a high-saturated fat diet combined with PSt enhanced the absorption of SFAs (C12:0, C14:0, C16:0) in guinea pigs [53, 54]. According to Brufau et al. [53], an increased absorption of FAs is indicated by elevated plasma SFA and MUFA proportions following combined high-dose PL + PSt supplementation in this study. Moreover, increased plasma SM proportions together with decreased PC proportion, a slightly decreased fecal fat excretion rate, a reduced fecal MUFA fraction, and a decreased fecal total PL concentration point to an increased fat absorption, in particular of FAs or FA-containing intestinal hydrolysis products during consumption of 6 g PL + 2 g PSt compared with 6 g PL.

Free FAs and FA-containing hydrolysis products (e.g., lyso-PC, monoglycerides) are generated by luminal enzymes during intestinal emulsification [9, 44]. The FAs and hydrolysis products are mostly dissolved and imbedded in luminal mixed micelles, which are formed in the presence of biliary PLs, cholesterol, and bile acids. Additionally, specific ratios of micelle constituents are necessary for dissolving apolar lipids [55]. Therefore, increased fat absorption following supplementation of PL combined with PSt could possibly due to an improved intestinal condition for originating luminal emulsions and mixed micelles following PL and sterol, more precisely, PSt supplementation compared with only PL supplementation. An improved intestinal condition for emulsification might also result in a higher enzymatic generation of FAs and FA-containing hydrolysis products. However, in vitro and in vivo studies reveal some membrane proteins, for example, scavenger receptor class B type I (SR-BI), cluster of differentiation 36 (CD 36), and membrane fatty acid binding protein (FABP) as being associated with FA, PL, TG, and/or cholesterol uptake [56–60]. Additionally, in vivo studies demonstrated that PSts may effect the expression of SR-BI and CD36 [61, 62]. However, whether the presence of a high milk PL dose combined with the cholesterol-like compound PSt influences the diverse transport functions of SR-BI, CD36, or FABP is not known, to date.

In summary, supplementation with a moderate and with a high dose of milk PL resulted mainly in changes in FA distribution in the plasma of healthy volunteers. No significant changes in LDL cholesterol concentration were found following moderate PL supplementation, whereby a high dose of milk PL resulted in raised LDL cholesterol compared with moderately dosed supplementation. The LDL/HDL ratio of cholesterol was unchanged during the course of the study. Compared with exclusive high-dose PL supplementation, administration of high-dose PL + PSt led to a decreased plasma LDL cholesterol concentration, increased plasma SM/PC ratio, increased plasma SFA and MUFA proportions, and decreased PL excretion, due probably to an enforced absorption of FAs or FA-containing hydrolysis products that originate during lipid digestion.

Acknowledgments We would like to thank our volunteers for their engaged participation in the study. Nasim Kroegel is acknowledged for language editing. This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 316ZBG.

Conflict of interest None.

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